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(21) International Application Number: PCT/US96/01257 (22) International Filing Date: 26 January 1996 (26.01.96) (30) Priority Data: 08/379,851 27 January 1995 (27.01.95) US (71) Applicant: THE GOVERNMENT OF THE UNITED STATES OF AMERICA, represented by THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES (US/US); National Institutes of Health, Office of Technology Transfer, Box OTT, Bethesda, MD 20892-9902 (US). (72) Inventors: HENEINE, Walid; 2001 Hollidon Road, Decatur, GA 30333 (US). FOLKS, Thomas, M.; 3815 Belle Glade Trail, Lithonia, GA 30058 (US). SWITZER, William, Marshall; 820 Stephenson Ridge, Stone Mountain, GA 30087 (US). YAMAMOTO, Shinji; 3131 North Druid Hills Road, Decatur, GA 30033 (US). (74) Agents: NEEDLE, William, H. et al.; Needle & Rosenberg, The Candler Building, Suite 1200, 127 Peachtree Street, N.E., Atlanta, GA 30303-1811 (US).		(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: METHODS FOR SENSITIVE DETECTION OF REVERSE TRANSCRIPTASE (57) Abstract The present invention provides a method for detecting the presence of a retrovirus in a biological sample comprising the steps of: a) contacting the biological sample with an RNA template and a complementary DNA primer under conditions whereby the RNA template and the DNA primer will anneal and a DNA strand will be synthesized as an extension from the DNA primer if reverse transcriptase is present in the sample; b) amplifying the synthesized DNA; and c) detecting the amplification of the synthesized DNA, the amplification of the synthesized DNA indicating the presence of reverse transcriptase in the biological sample, thus indicating the presence of a retrovirus in the biological sample.		

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METHODS FOR SENSITIVE DETECTION OF REVERSE TRANSCRIPTASE

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BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

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The present invention provides a method for detecting the presence of a retrovirus in a biological sample by detecting the presence of the enzyme, reverse transcriptase. The method utilizes a template and primers in conditions under which a DNA strand is synthesized and subsequently amplified by the polymerase chain reaction, if reverse transcriptase is present.

15

BACKGROUND ART

Retroviruses are widely distributed in vertebrates and are known to cause a variety of diseases in man and animals including immunodeficiencies, leukemias and lymphomas (1). The entire retrovirus family is characterized by the presence of a unique enzyme, reverse transcriptase (RT), which transcribes the viral genomic RNA into a double-stranded DNA copy (1). This feature has led to studies of the unique enzymatic function of RT for two main applications. First, the presence of RT has been the basis for diagnosis of retroviral infection and for the generic detection of the presence of retroviruses in cell cultures and in the infected host. Second, the RT enzyme constitutes a primary target for antiviral drug intervention (1,2).

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The polymerase function of RT from several retroviruses has been well characterized and shown to require an RNA template, a primer, triphosphate, a divalent cation and physiological salts (1). Therefore, assays for RT activity have conventionally used these reagents and conditions to measure the ability of a sample to produce a DNA
5 copy of a known exogenous RNA template (e.g., poly rA) by synthesizing a complementary DNA oligonucleotide primer with radiolabeled nucleotides (e.g., ^3H - or ^{32}P -dTTP). RT synthesized DNA has been detected by measuring incorporation of the labeled nucleotide (3,4) and more recently, by assays which employ nonradioactive nucleotides in enzyme linked immunosorbent assay (ELISA) formats have been
10 developed (5-8) and by polymerase chain reaction (PCR) (17), as further described below.

Although RT assays continue to be an essential laboratory tool for the identification of known and novel retroviruses (4-11), the successful use of RT assays
15 has been limited to the detection of retroviral particles in culture supernatant (4-8), which has the disadvantage of requiring that a virus be cultured before detection. By the present methods, lentiviruses, such as human immunodeficiency virus-1 (HIV-1) may be readily detected, but oncoviruses, such as human T lymphocytic virus types I and II (HTLV-I and HTLV-II), are much more difficult to detect, presumably because of poor
20 RT activity and because they are typically cell-associated, which means their RT is less accessible for detection in culture supernatants (6-8). This limitation has rendered RT testing of little value in the detection of HTLV infection.

Despite multiple attempts to improve the sensitivity of RT assays, the direct detection of retroviruses in clinical samples (e.g., serum) has been unsuccessful (4-12). For example, in studies of HIV-1 infected individuals, detection of virus in plasma by RT assays has been largely abandoned because of the low sensitivity of this method (12).

5

The inability to detect RT activity in serum hinders the use of this virological marker in diagnosing disease, monitoring drug efficacy in patients, monitoring virus load and predicting disease progression. Present methods for qualitative and quantitative detection of plasma HIV-1 include viral isolation and p24 antigen capture (13-15). However, these assays have disadvantages. Virus isolation from plasma requires virus culture that is typically maintained for 14 to 28 days and is labor intensive, time consuming, fraught with biological variation and is not a universal marker in the infected population given the low levels of HIV-1 in patients with CD4+ T lymphocyte counts of $>200/\text{mm}^3$. Similarly, p24 antigen, either free, virion-associated, or immune complexed, is not always present in the HIV-1 infected population.

RT-PCR permits the qualitative detection of the cell-free virus in plasma using an exogenous RT and a primer pair of known sequence to amplify viral RNA sequences in the plasma (15,16). Although RT-PCR has been reported to be highly sensitive, this assay requires RNA extraction and multiple sample manipulations that may increase the risks of PCR contamination. The RT-PCR assay may be complicated further by the lack of a standardized universal quantitative test and the variabilities that may be incurred during processing and storage with regard to the degradation of the genomic RNA. Although RT-PCR, like antigen capture, is highly specific, a knowledge of the

nucleotide sequence of the target retrovirus fragment is necessary for primer development. Given this limitation, RT-PCR is not suitable for detecting variant, novel, or unknown retroviruses.

5 Recently, two assays for RT, which use the RNA of bacteriophage MS2 or brome mosaic virus (BMV) as a template and PCR as a detection system, have been reported to be highly sensitive for the detection of murine leukemia virus RT and other stocks of retroviruses in serum (18,19). However, the evaluation of the specificity and the sensitivity of these assays on adequate numbers of serum specimens was not
10 disclosed (18,19). In addition, in both of these assays, problems with inhibitors of RT activity in serum and nonspecific background RT activity have been described (20), raising serious questions about the diagnostic value of these assays.

 This invention provides a RT assay that employs a PCR-based amplification
15 system to detect a known cDNA product of the RT reaction. The assay of the present invention, referred to hereafter as Amp-RT, is highly sensitive and specific, requires no knowledge of viral genomic sequence and allows the detection of RT activity in samples of individuals infected with retroviruses or any other biological entity that produces RT.

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SUMMARY OF THE INVENTION

 The present invention provides a method for detecting the presence of a retrovirus in a biological sample comprising the steps of: a) contacting the biological

sample with an RNA template and a complementary DNA primer under conditions whereby the RNA template and the DNA primer will anneal and a DNA strand will be synthesized as an extension from the DNA primer if reverse transcriptase is present in the sample; b) amplifying the synthesized DNA; and c) detecting the amplification of the synthesized DNA, the amplification of the synthesized DNA indicating the presence of reverse transcriptase in the biological sample, thus indicating the presence of a retrovirus in the biological sample.

The present invention also provides a method of detecting the presence of a retrovirus in a biological sample comprising the steps of: a) contacting the biological sample with an RNA template, wherein the RNA template is a suitable region of the encephalomyocarditis virus which consists of the ribonucleotide of SEQ ID NO:4 and a complementary DNA primer, wherein the primer is the oligonucleotide of SEQ ID NO:2, under conditions whereby the RNA template and the DNA primer will anneal and a DNA strand will be synthesized as an extension from the DNA primer if reverse transcriptase is present in the sample; b) amplifying the synthesized DNA, wherein the amplification is by the polymerase chain reaction method whereby the conditions for the amplification comprise 30-40 cycles of heating the synthesized DNA and a primer pair to 93 to 96°C for 30 seconds to one minute, 53 to 56°C for 30 seconds to one minute and 70 to 74°C for 30 seconds to five minutes, wherein the primer pair consists of the oligonucleotide consisting essentially of SEQ ID NO:1 and the oligonucleotide consisting essentially of SEQ ID NO:2; and c) detecting the amplification of the synthesized DNA, wherein the detection of the amplification of the synthesized DNA is by Southern blot hybridization assay, with a probe consisting essentially of the

oligonucleotide of SEQ ID NO:3, the amplification of the synthesized DNA indicating the presence of reverse transcriptase in the biological sample, thus indicating the presence of a retrovirus in the biological sample.

5 Additionally provided is a kit for detecting the presence of a retrovirus in a biological sample, comprising a suitable region of the encephalomyocarditis virus genome as an RNA template and a complementary DNA primer for reverse transcriptase and a primer pair for polymerase chain reaction, whereby each component is provided in separate containers or any combination of the components is provided in a single
10 container.

DETAILED DESCRIPTION OF THE INVENTION

As used in the specification and in the claims, "a" can mean one or more,
15 depending on the context in which it is used.

The present invention provides a method for detecting the presence of a retrovirus in a biological sample comprising the steps of: a) contacting the biological sample with an RNA template and a complementary DNA primer under conditions
20 whereby the RNA template and the DNA primer will anneal and a DNA strand will be synthesized as an extension from the DNA primer if reverse transcriptase is present in the sample; b) amplifying the synthesized DNA; and c) detecting the amplification of the synthesized DNA, the amplification of the synthesized DNA indicating the presence of

reverse transcriptase in the biological sample, thus indicating the presence of a retrovirus in the biological sample.

The RNA template can comprise a suitable region of any ribonucleotide sequence such as, for example, the encephalomyocarditis virus genome. As used herein, a "suitable region" means a region of the RNA sequence having no significant secondary structure, less than 50% G-C content and to which complementary DNA primers can be generated which have T_m values within the range of reaction temperatures appropriate for the synthesis of a DNA strand, as further described herein. The RNA template can be of a length sufficient to produce a DNA product ranging in size from 100 to 500 base pairs in length and most preferably about 300 base pairs in length. The RNA template can be the ribonucleotide of SEQ ID NO:4.

As used herein, "complementary DNA primer" means an oligonucleotide which anneals to the RNA template in a particular orientation to allow for the synthesis of a nascent DNA strand in the presence of RT in the biological sample under the conditions described herein. Also as used herein, the "conditions" under which a DNA strand is synthesized include the presence of nucleotides, cations and appropriate buffering agents in amounts and at temperatures such that the RNA template and the DNA primer will anneal and oligonucleotides will be incorporated into a synthesized DNA strand if reverse transcriptase is present. More specifically, an example of these conditions is provided in the Examples section. The described conditions have been optimized from other known RT/cDNA synthesis protocols. It is generally known that other conditions can be established for optimization of a particular RT reaction on the basis of protocols

well known to one of ordinary skill in the art. The DNA primer can be the reverse primer of a primer pair to be used in a subsequent amplification by PCR, such as, for example, the oligonucleotide of SEQ ID NO:2 (EMCR2).

5 The biological sample can comprise any biological tissue or body fluid (e.g., cells, serum, plasma, semen, urine, saliva, sputum, cerebrospinal fluid). The synthesized DNA strand can be amplified by any of the amplification protocols known in the art now or in the future, including but not limited to the polymerase chain reaction (PCR) (17), the ligation amplification reaction (LAR) (21), the ligase-based amplification system
10 (LAS) (22), the self-sustained sequence replication (3SR) system (23), the transcription-based amplification system (TAS) (24) and the Q β replicase amplification method (25).

For amplification of the synthesized DNA by PCR, the conditions for amplification can include 30 to 40 (most preferably 35) cycles of heating the synthesized
15 DNA and a primer pair to 93° to 96°C (most preferably 95°C) for 30 seconds to one minute (most preferably one minute), 53° to 56°C (most preferably 55°C) for 30 seconds to one minute (most preferably one minute) and 70° to 74°C (most preferably 72°C) for 30 seconds to five minutes (most preferably one minute).

20 As used herein, "a primer pair" refers to two primers, one having a forward designation and the other having a reverse designation relative to their respective orientations on a double-stranded DNA molecule which consists of a sense and antisense sequence, such that under the amplification conditions described herein, the forward primer anneals to and primes amplification of the sense sequence and the reverse primer

anneals to and primes amplification of the antisense sequence. Primers can be selected for use in the amplification reaction on the basis of having less than 50% G-C content, having minimal complementarity with other primers in the reaction (to minimize the formation of primer dimers) and having T_m values within the range of reaction

5 temperatures appropriate for PCR. In addition, primers can be selected to anneal with specific regions of the RNA template such that the resulting DNA amplification product ranges in size from 100 to 500 base pairs in length and most preferably around 300 base pairs in length. For example, in the conditions described above, the primer pair can consist of the oligonucleotide of SEQ ID NO:1 (EMCF1) as the forward primer and the

10 oligonucleotide of SEQ ID NO:2 (EMCR2) as the reverse primer.

As used herein, "detecting" or "detection" of the amplified DNA refers to quantitatively or qualitatively determining the presence of the amplified DNA strand which is only synthesized if RT is present in the biological sample. The amplification of

15 the synthesized DNA can be detected by any method for the detection of DNA known in the art. For example, detection of the amplified DNA can be by Southern blot hybridization assay, by visualization of PCR products of specific molecular weight on ethidium bromide stained agarose gels, by measurement of the incorporation of radiolabeled nucleotides into the synthesized DNA strand by autoradiography or

20 scintillation measurement, by ELISA modified for the capture of a detectable moiety bound to the amplified DNA, or any other detection method known to one of ordinary skill in the art.

For detection by Southern blot hybridization assay, the synthesized DNA can be detected by a probe specific for the DNA synthesized from the template. For example, such a specific probe can consist essentially of the oligonucleotide of SEQ ID NO:3 (EMCP1). A Southern blot hybridization protocol for use in the invention is

5 demonstrated in the Examples.

The present invention provides a method for detecting the presence of a retrovirus in a biological sample comprising the steps of: a) contacting the biological sample with a suitable region of the encephalomyocarditis virus genome as an RNA

10 template and a complementary DNA primer under conditions whereby the RNA template and the DNA primer will anneal and a DNA strand will be synthesized as an extension from the DNA primer if reverse transcriptase is present in the sample;

b) amplifying the synthesized DNA; and c) detecting the amplification of the synthesized DNA, the amplification of the synthesized DNA indicating the presence of reverse

15 transcriptase in the biological sample, thus indicating the presence of a retrovirus in the biological sample.

The present invention also provides a method of detecting the presence of a retrovirus in a biological sample comprising the steps of: a) contacting the biological

20 sample with an RNA template and a complementary DNA primer under conditions whereby the RNA template and the DNA primer will anneal and a DNA strand will be synthesized as an extension from the DNA primer if reverse transcriptase is present in the sample; b) amplifying the synthesized DNA by the polymerase chain reaction method whereby the conditions for the amplification comprise 30-40 cycles of heating the

synthesized DNA and a primer pair to 93 to 96°C for 30 seconds to one minute, 53 to 56°C for 30 seconds to one minute and 70 to 74°C for 30 seconds to five minutes; and c) detecting the amplification of the synthesized DNA, the amplification of the synthesized DNA indicating the presence of reverse transcriptase in the biological sample, thus indicating the presence of a retrovirus in the biological sample.

The present invention further provides a method for detecting the presence of a retrovirus in a biological sample comprising the steps of: a) contacting the biological sample with a region of the encephalomyocarditis virus genome as an RNA template and a complementary DNA primer under conditions whereby the RNA template and the DNA primer will anneal and a DNA strand will be synthesized as an extension from the DNA primer if reverse transcriptase is present in the sample; b) amplifying the synthesized DNA by the polymerase chain reaction method whereby the conditions for the amplification comprise 30-40 cycles of heating the synthesized DNA and a primer pair to 93 to 96°C for 30 seconds to one minute, 53 to 56°C for 30 seconds to one minute and 70 to 74°C for 30 seconds to five minutes; and c) detecting the amplification of the synthesized DNA, the amplification of the synthesized DNA indicating the presence of reverse transcriptase in the biological sample, thus indicating the presence of a retrovirus in the biological sample.

As an example, the present invention can provide a method for detecting a retrovirus in a biological sample comprising the steps of: a) contacting the biological sample with a suitable region of the encephalomyocarditis virus genome as an RNA template, wherein the RNA template is the ribonucleotide of SEQ ID NO:4 and a

complementary DNA primer, wherein the primer is the oligonucleotide of SEQ ID NO:2, under conditions whereby the RNA template and the DNA primer will anneal and a DNA strand will be synthesized as an extension from the DNA primer if reverse transcriptase is present in the sample; b) amplifying the synthesized DNA, wherein the DNA is amplified by the polymerase chain reaction under conditions which comprise about 35 cycles of heating the synthesized DNA and a primer pair to 95°C for one minute, 55°C for one minute and 72°C for one minute, wherein the primer pair consists of the oligonucleotide consisting essentially of the SEQ ID NO:1 and the oligonucleotide consisting essentially of the SEQ ID NO:2; and c) detecting the amplification of the synthesized DNA, wherein the amplification is detected by Southern blot hybridization assay with a probe consisting essentially of the oligonucleotide of SEQ ID NO:3.

Additionally provided is a kit for detecting the presence of a retrovirus in a biological sample, comprising the enzymes, buffering agents, cations and oligonucleotides well known in the art for carrying out RT and PCR reactions. The kit also comprises a suitable region of the encephalomyocarditis virus genome as an RNA template and a complementary DNA primer for reverse transcriptase and a primer pair for polymerase chain reaction. The RNA template, the complementary DNA primer and the primers of the primer pair can each be in separate containers or all or any combination of these components can be combined in a single container. The complementary DNA primer can be the oligonucleotide of SEQ ID NO:2 (EMCR2), the RNA template can be the ribonucleotide of SEQ ID NO:4 and the primer pair can

consist of the oligonucleotide consisting essentially of SEQ ID NO:1 (EMCF1) and the oligonucleotide consisting essentially of SEQ ID NO:2 (EMCR2).

The present invention has additional applications based on the basic inventive principle that RT can be detected and quantitated in a biological sample. One application of this principle is the differential identification of retroviruses on the basis of the specific reactivity of the detected RT with antibodies against different retroviral RTs (for example, antibodies which can distinguish RT produced by HIV-1 or HIV-2 in infected individuals). Each retrovirus has a distinct RT, to which specific antibodies can be generated, thereby permitting identification of the specific retrovirus. The method of the present invention can be employed to first detect the presence of RT and various antibodies specific for RTs of different retroviruses can then be added to the biological sample to identify the type of retrovirus present. An antibody of known RT specificity will bind the RT present in the biological sample if the RT is produced by the virus to which the antibody is specific and will inhibit its activity, resulting in no DNA synthesis in the presence of antibody.

Another application of the present invention is screening patients for resistance to drug therapy. The susceptibility of RT to anti-RT drugs can be monitored over time in a patient receiving anti-RT drug therapy. The present invention can be employed to detect the emergence of anti-RT drug resistance in a patient by direct testing of the patient's serum for RT activity as an indicator of the susceptibility of the RT in the patient's serum to the anti-RT drug(s) used. If drug resistance is detected, alternative treatment strategies may be implemented. This invention obviates the need for the

lengthy and labor-intensive culture methods currently used to study drug resistance in patients.

A third application of the present invention is the monitoring of virus load in patients infected with biological entities which produce RT. Quantitative measurement of RT over time can be correlated with survival and/or recovery rates in patients with illnesses caused by these entities, for the purpose of following disease progression and prognosing the patient's illness.

The present invention is more particularly described in the following examples which are intended as illustrative only since numerous modifications and variations therein will be apparent to those skilled in the art.

EXAMPLES

15

The following examples are intended to illustrate, but not limit, the invention. While the protocols described are typical of those that might be used, other procedures known to those skilled in the art may be alternatively employed.

20 Viruses

The retroviruses used in this study were prepared as follows. HIV-1 Lai and simian immunodeficiency virus (SIVB670) were propagated in peripheral blood lymphocytes (PBLs). Caprine arthritis encephalitis virus (CAEV-63) was propagated in fetal goat synovial membrane cells. HTLV-I and HTLV-II were obtained from

supernatants of MT-2 and Mo-T cell lines; simian retroviruses types 1 and 2 (SRV-1 and SRV-2) were grown in Raji cells; gibbon ape leukemia virus (GALV) was grown in Jurkat cells and simian foamy virus type 3 (SFV-3) was grown in the Cf2Th cell line.

5 ***In vitro* transcription of the RNA template**

An RNA sequence from the encephalomyocarditis virus (EMCV) was used as template and was generated from a plasmid vector obtained from Novagen (Madison, WI, USA). A small EMCV sequence (350 bp) (SEQ ID NO:4) was amplified from the plasmid, using standard PCR conditions and the primer pair T7-EMCF1 (SEQ ID NO:6) and EMCR2 (SEQ ID NO:2). The sequence of T7-EMCF1 (SEQ ID NO:6) was:
5'GGTACCTAATACGACTCACTATAGGGAGACATTAGCCATTTC AACCCAT3'
and that of EMCR2 (SEQ ID NO:2) was:
5'GTTTCATGACAGGCCGATACAGAGG3'.

15 To allow the *in vitro* transcription of this PCR product, the sense primer, EMCF1: 5'CATTAGCCATTTC AACCCAT3' (SEQ ID NO:1), was modified at the 5' end by adding a T7 promoter sequence: 5'GGTACCTAATACGACTCACTAT-3' (SEQ ID NO:5).

20 The EMCV-amplified product was then transcribed with the large scale T7 transcription kit from Novagen according to the manufacturer's instructions. The DNA in the RNA preparation was subsequently digested twice with 20 units of RNase-free DNase (Promega) at 37°C for 60 minutes and the DNase was inactivated by heating at 95°C for ten minutes. The purity of the RNA preparation was checked for residual DNA

contamination by PCR amplification with EMCF1 (SEQ ID NO:1) and EMCR2 (SEQ ID NO:2) and subsequent Southern blot hybridization to the ³²P-end-labeled internal probe EMCP1 (SEQ ID NO:3): 5'TGCTCTCACCTTATCAAAATCCAAT3'.

5 **Amp-RT**

Twenty ul or less of the biological sample to be tested was added to 40 ul of RT buffer containing 10 ng of RNA template, 10 units of RNasin, 0.06% NP-40, 200 ng primer (EMCR2, SEQ ID NO:2), 0.8mM EGTA, 2mM DTT, 50mM Tris-HCl, 50mM KCl, and 10mM MgCl₂. The reaction was incubated at 37° C for two hours and then
10 heated at 95°C for ten minutes to inactivate any RT activity. A volume of 50 ul of standard PCR buffer (17) containing 0.5 units of Taq polymerase and 200 ng of the sense primer without the T7 sequence (EMCF1, SEQ ID NO:1) was added to this mixture. The reaction was cycled 35 times at 95°C for one minute, 55°C for one minute and 72°C for one minute. Twenty ul of the reaction product was electrophoresed in
15 1.8% agarose gels and Southern blot hybridized to a ³²P-end-labeled internal probe (EMCP1, SEQ ID NO:3) at 42°C overnight. The blots were washed in 2X SSC (NaCl and sodium citrate), 0.5% sodium dodecyl sulfate (SDS) for one to two hours. The blots were exposed from six to 24 hours.

20 To check the integrity and the purity of the RNA template preparation, RT reactions were carried out using the EMCV RNA template (SEQ ID NO:4) and an HIV-1 virus stock as the RT source. While the HIV-1 reactions were positive, control reactions which had no HIV-1, or which were pretreated with RNase before the addition of HIV-1, were all negative. These negative results indicated that the underlying

reaction of Amp-RT was RNA-dependent and that the RNA template and other assay components were free of any contaminating target DNA. To further confirm that the reaction was mediated by RT, Amp-RT reactions were prepared by using HIV-1 culture supernatant in the presence of 2 ug of tetrahydroimidazo-benzodiazepin (TIBO)

- 5 compounds, which are non-nucleoside RT inhibitors (2). The results demonstrated inhibition of the Amp-RT reaction only in the presence of the TIBO compounds, while the DNA polymerase activity of Taq in the control amplification reactions was not affected.

10 **Comparative sensitivity analysis of Amp-RT**

Many specific and generic assays are currently used to detect the presence of retroviruses. To compare the relative sensitivities of these assays to Amp-RT, serial 10-fold dilutions of an HIV-1 culture supernatant were made in culture medium and then divided into equal aliquots. Separate aliquots were subjected to testing by standard

15 RT assay, branched DNA detection, p24 antigen capture, 50% tissue culture infective dose (TCID₅₀) determination, RT-PCR and Amp-RT.

Standard RT assay

- RT activity was measured in culture supernatant with the template primer of
- 20 poly(rA).oligo dT according to the methods of Willey et al. (3). The enzymatic activity was assessed by measuring the incorporated tritiated thymidine monophosphate.

Branched DNA (bdNA) detection of HIV-1

Branched DNA (bDNA) detection of HIV- 1 was performed according to the manufacturer's instructions (Chiron, Emryville, California, USA). A cutoff of 5,000 RNA equivalents/ml was used, as recommended by the manufacturer.

5

p24 antigen capture

Levels of base dissociated-p24 antigens of HIV-1 were determined by a commercially available ELISA kit from Organon Technika (North Carolina, USA).

10 **TCID₅₀ determination**

The TCID₅₀ of the HIV-1 viral stock was determined on PBLs as previously described by McDougal et al. (26).

RT-PCR

15 Particle-associated HIV-1 genomic RNA in culture supernatant was extracted with phenol/chloroform, precipitated with ethanol and reconstituted in 40 ul of RT buffer [50mM Tris-Cl (pH 8.3), 20mM KCl, 10mM MgCl₂] (15). RNA was further digested with 5 units of DNase-I (Promega) in the presence of 10mM sodium acetate at 37°C for 30 minutes. DNase was inactivated by heating at 95°C for ten minutes. For
20 reverse transcription, 10 ul of RNA was added to 20 ul of the RT mixture, containing 10mM DTT, 20 units RNasin, 0.875mM each of GTP, ATP, CTP and TTP, 200 ng of reverse primer (SK39, SEQ ID NO:8) and 0.02 units of AMV-reverse transcriptase. The mixture was reverse transcribed at 42°C for 45 minutes. Controls included 10 ul aliquots that were not reverse transcribed. All samples were then amplified by PCR in

100 ul reaction volumes for 35 cycles, using SK38/SK39 (SEQ ID NOS:7 and 8) as primers and the amplified products were Southern blot hybridized to the ³²P-labeled SK19 (SEQ ID NO:9) probe (27).

5 The results, shown in Table 1, demonstrated that Amp-RT was the most sensitive assay and was 100,000 times more sensitive than the standard RT assay, 10,000 times more sensitive than the bDNA detection system and the p24 antigen capture assay and 100 times more sensitive than TCID₅₀ and RT-PCR. The p24 concentration in the undiluted HIV-1 sample was found to be 1.6 ng.

10

Testing of clinical samples with Amp-RT

Forty-two serum samples from HIV-1 seropositive individuals enrolled in a study of the natural history of HIV in homosexual men in Atlanta, Georgia, USA (28) were tested by Amp-RT. The clinical stage of the HIV-1 infection in these men was
15 determined on the basis of the Centers for Disease Control and Prevention (CDC) revised classification system (29). Laboratory markers, including CD4+ lymphocyte counts and percentage of CD4+ lymphocytes in the blood, were determined by standard flow cytometric methods. Serum samples from 20 healthy individuals who were HTLV-I/II and HIV-1 seronegative were included as controls.

20

Preliminary experiments using normal human serum spiked with HIV-1 were all Amp-RT negative and indicated that preparation of serum samples for Amp-RT requires special conditions different from those described for culture supernatant. For instance, even small volumes (5-20 ul) of serum or plasma could not be directly tested by

Amp-RT because of protein precipitation that develops during the high temperature stage of the PCR cycle. Therefore, to avoid this problem, sera were ultracentrifuged and the Amp-RT assay was performed using the viral pellet.

- 5 For testing of culture supernatant, 20 ul or less was used for the RT reaction. For testing serum or plasma samples, 0.5 ml was diluted 10-fold in DEPC-treated phosphate buffered saline, clarified by centrifugation at 1,000 g for 10 minutes and then ultracentrifuged at 44,000 g for one hour. The pellet was suspended in 50 ul of RT buffer containing 0.6% NP-40 for 15 minutes and aliquots of 5-45 ul were used in the
10 Amp-RT assay.

- The results of testing serum samples indicated that Amp-RT was able to detect RT activity in 36 (85.7%) serum samples (Table 2), 31 of which (86.1%) had detectable p24 antigen. This high sensitivity was also coupled with a high specificity since none of
15 the HIV-1/HTLV seronegative samples were positive. Of the six samples from patients classified as A1, three were Amp-RT positive (50%), while five of seven (71.4%) and 25 of 26 (96.1%) of the samples from patients classified as B2 and C3, respectively, were Amp-RT positive.

20 **Testing of the hepatitis B virus (HBV) by Amp-RT**

Because the polymerase gene of HBV has been reported to possess RT-like activity (30), Amp-RT was employed to detect the HBV-associated RT activity. Serum samples from 21 HBV-infected individuals were selected, including 11 samples with

detectable HBe antigen (Abbott HBe EIA, Chicago, USA) and ten other samples with no detectable HBe antigen.

Despite the documented presence of HBV virions in 11 serum specimens, none of the samples yielded positive results by Amp-RT analysis. These negative results exclude the possibility of the HBV-associated RT interfering in the Amp-RT assay, at least under these conditions, and therefore confirm the specificity range of the assay for the detection of only retroviral RTs.

10 **Detection of different retroviruses by Amp-RT.**

The ability of Amp-RT to detect a wide variety of retroviruses representing members of all three retroviral subfamilies was investigated. The lentiviruses tested included HIV-1, SIV and CAEV. HTLV-I, HTLV-II, GALV, SRV-1 and SRV-2 were representative of the oncoviruses and SFV-3 was representative of the spumaviruses.

15 Amp-RT was capable of detecting all retroviruses tested. Most importantly, viruses such as HTLV-I, HTLV-II and GALV, which are typically difficult to detect with the standard RT methodology, were easily identified with this assay.

These viruses could be detected by Amp-RT in dilutions containing 0.04 to 0.00004 ul of the original unconcentrated culture supernatant. The serial dilutions of the tested retroviruses were made in supernatant from uninfected cell lines (Hut-78, A301 or U937). All three supernatants consistently tested negative, as can be seen from the end point dilutions of the tested retroviruses.

Side by side comparison of previously published RT assays

To demonstrate that the assay of the present invention provides increased specificity and sensitivity in comparison to other published RT assays [22,23], a side by side comparison of these assays can be performed. These assays differ in the particular
5 RNA template sequence, primer sequences, RT buffer compositions, amplification conditions, detection conditions and sample preparation used. Therefore, for a comparison of the two assays, a panel of serum samples from HIV-1 seropositive individuals and from HIV-1 and HTLV-I/II negative individuals can be tested according to the teachings of the present assay and each other assay. Positive controls can include
10 normal human serum spiked with HIV-1 virus particles. The specificity and sensitivity of these assays can then be computed and compared.

Throughout this application various publications are referenced by numbers within parentheses. Full citations for these publications are as follows. The disclosures
15 of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

Although the present process has been described with reference to specific
20 details of certain embodiments thereof, it is not intended that such details should be regarded as limitations upon the scope of the invention except as and to the extent that they are included in the accompanying claims.

Table 1. Comparison of the sensitivity of Amp-RT in the detection of HIV-1 with other generic and virus-specific methods.

HIV-1 dilution	Std. RT	bDNA	p24 ag	TCID ₅₀	RT-PCR	Amp-RT
10 ⁰	+	+	+	+	+	+
10 ⁻¹	+	+	+	+	+	+
10 ⁻²	+	+	+	+	+	+
10 ⁻³	-	+	+	+	+	+
10 ⁻⁴	-	-	-	+	+	+
10 ⁻⁵	-	-	-	+	+	+
10 ⁻⁶	-	-	-	-	-	+
10 ⁻⁷	-	-	-	-	-	+
10 ⁻⁸	-	-	-	-	-	-

Table 2. Detection of RT activity by Amp-RT in serum samples from HIV-1 infected individuals characterized by clinical stage, CD4+ lymphocyte counts in the peripheral blood and amount of p24 antigen in the serum.

<u>No</u>	<u>Patient ID</u>	<u>p24 (pg/ml)</u>	<u>%CD4</u>	<u>CD4 count</u>	<u>Clinical Stage*</u>	<u>Amp-RT</u>
1	69877	NEG	27	830	A1	NEG
2	82850	NEG	23	699	A1	P
3	69878	141	33	566	A1	P
4	77913	NEG	25	599	A1	P
5	111380	49	27	778	A1	NEG
6	111318	49	28	539	A1	NEG
7	85987	141	23	348	A2	P
8	66583	55	13	286	A2	P
9	62303	42	12	341	B2	P
10	71172	56	22	281	B2	NEG
11	71245	100	11	208	B2	P
12	77149	44	16	282	B2	P
13	86563	141	14	286	B2	P
14	66994	NEG	25	260	B2	NEG
15	57589	141	18	370	B2	P
16	21149	NEG	10	97	B3	P
17	70145	8	5	26	C3	NEG
18	84300	141	1	4	C3	P
19	111207	N	10	103	C3	P
20	67365	141	5	24	C3	P
21	96215	40	6	80	C3	P
22	120979	141	3	19	C3	P
23	20525	73	3	22	C3	P
24	736	103	8	50	C3	P
25	1837	158	3	6	C3	P
26	3125	150	5	12	C3	P

25

27	3369	33	1	1	C3	P
28	20577	85	6	123	C3	P
29	20525	73	3	22	C3	P
30	20577	85	6	123	C3	P
31	20709	158	10	42	C3	P
32	20876	80	7	57	C3	P
33	21156	124	14	106	C3	P
34	21196	24	8	48	C3	P
35	21684	45	4	25	C3	P
36	23505	64	4	19	C3	P
37	23507	15	3	52	C3	P
38	24462	149	6	41	C3	P
39	24705	149	5	128	C3	P
40	25238	98	5	13	C3	P
41	23209	71	5	10	C3	P
42	53416	NEG	14	81	C3	P

*A1 and A2 refer to asymptomatic, acute (primary) or persistent generalized lymphadenopathy with CD4+ lymphocyte counts of >500/ul or 200-499/ul, respectively. B2 and B3 refer to symptomatic conditions different from those in stage A or AIDS, with CD4+ lymphocyte counts of 200-499/ul and <200ul, respectively. C3 refers to AIDS, with CD4+ lymphocyte counts of <200/ul (29).

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: HENEINE, WALID
FOLKS, THOMAS M.
SWITZER, WILLIAM M.
YAMAMOTO, SHINJI
- (ii) TITLE OF INVENTION: METHODS FOR SENSITIVE DETECTION OF
REVERSE TRANSCRIPTASE
- (iii) NUMBER OF SEQUENCES: 9
- (iv) CORRESPONDENCE ADDRESS:
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 - (C) CITY: Atlanta
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 - (F) ZIP: 30303
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Spratt, Gwendolyn D.
 - (B) REGISTRATION NUMBER: 36,016
 - (C) REFERENCE/DOCKET NUMBER: 1414.628
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 404/688-0770
 - (B) TELEFAX: 404/688-9880

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CATTAGCCAT TTCAACCCAT

20

(2) INFORMATION FOR SEQ ID NO:2:

31

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GTTCATGACA GGCCGATACA GAGG

24

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 25 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TGCTCTCACC TTATCAAAAT CCAAT

25

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 374 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: RNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CAUUAGCCAU UUCAACCCAU GCGUUUGAGG AGAAGCGCUU UCUGAUAACC GGUGGUCUCC	60
CAUCAGGUUG UGCAGCGACC UCAAUGC UAA ACACUAUAAU GAAUAAUUAU AUAUUAGGG	120
CGGGUUUGUA UCUCACGUAU AAAAAUUUUG AAUUUGAUGA UGUGAAGGUG UUGUCGUACG	180
GAGAUGAUCU CCUUGUGGCC ACAAUUUACC AAUUGGAUUU UGAUAAGGUG AGAGCAAGCC	240
UCGCAAAGAC AGGAUUAUAG AUAACUCCCG CUAACACAAC UUCUACCUUU CCUCUAAUU	300
CGACGCUUGA AGACGUUGUC UUCUUAAAAA GAAAGUUUAA GAAAGAGGGC CCUCUGUAUC	360
GGCCUGUCAU GAAC	374

(2) INFORMATION FOR SEQ ID NO:5:

32

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 22 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGTACCTAAT ACGACTCACT AT

22

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 49 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGTACCTAAT ACGACTCACT ATAGGGAGAC ATTAGCCATT TCAACCCAT

49

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 28 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATAATCCACC TATCCCAGTA GGAGAAAT

28

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 28 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

33

TTTGGTCCTT GTCTTATGTC CAGAATGC

28

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATCCTGGGAT TAAATAAAAT AGTAAGAATG TATAGCCCTA C

41

What is claimed is:

1. A method for detecting the presence of a retrovirus in a biological sample comprising the steps of:
 - a) contacting the biological sample with a suitable region of the encephalomyocarditis virus genome as an RNA template and a complementary DNA primer under conditions whereby the RNA template and the DNA primer will anneal and a DNA strand will be synthesized as an extension from the DNA primer if reverse transcriptase is present in the sample;
 - b) amplifying the synthesized DNA; and
 - c) detecting the amplification of the synthesized DNA, the amplification of the synthesized DNA indicating the presence of reverse transcriptase in the biological sample, thus indicating the presence of a retrovirus in the biological sample.
2. The method of claim 1, wherein the complementary DNA primer is the oligonucleotide of SEQ ID NO:2.
3. The method of claim 1, wherein the synthesized DNA is amplified by the polymerase chain reaction.
4. The method of claim 3, wherein the conditions whereby the synthesized DNA is amplified comprise 30-40 cycles of heating the synthesized DNA and a primer pair to 93 to 96°C for 30 seconds to one minute, 53 to 56°C for 30 seconds to one minute and 70 to 74°C for 30 seconds to five minutes.
5. The method of claim 4, wherein the conditions whereby the synthesized DNA is amplified comprise about 35 cycles of heating the synthesized DNA and a primer pair to about 95°C for one minute, about 55°C for one minute and about 72°C for one minute.

6. The method of claim 5, wherein the primer pair consists of the oligonucleotide consisting essentially of SEQ ID NO:1 and the oligonucleotide consisting essentially of SEQ ID NO: 2.
7. The method of claim 1, wherein the RNA template is the ribonucleotide of SEQ ID NO:4.
8. The method of claim 1, wherein the amplification of the synthesized DNA is detected by Southern blot hybridization assay.
9. The method of claim 8, wherein the synthesized DNA is detected in the Southern blot hybridization assay with a probe consisting essentially of the oligonucleotide of SEQ ID NO:3.
10. A method of detecting the presence of a retrovirus in a biological sample comprising the steps of:
 - a) contacting the biological sample with an RNA template and a complementary DNA primer under conditions whereby the RNA template and the DNA primer will anneal and a DNA strand will be synthesized as an extension from the DNA primer if reverse transcriptase is present in the sample;
 - b) amplifying the synthesized DNA by the polymerase chain reaction method whereby the conditions for the amplification comprise 30-40 cycles of heating the synthesized DNA and a primer pair to 93 to 96°C for 30 seconds to one minute, 53 to 56°C for 30 seconds to one minute and 70 to 74°C for 30 seconds to five minutes; and
 - c) detecting the amplification of the synthesized DNA, the amplification of the synthesized DNA indicating the presence of reverse transcriptase in the biological sample, thus indicating the presence of a retrovirus in the biological sample.

11. The method of claim 10, wherein the RNA template is a region of the encephalomyocarditis virus genome.
12. The method of claim 11, wherein the RNA template is the ribonucleotide of SEQ ID NO:4.
13. The method of claim 10, wherein the conditions for the amplification of the DNA comprise about 35 cycles of heating the synthesized DNA and a primer pair to about 95°C for one minute, about 55°C for one minute and about 72°C for one minute.
14. The method of claim 13, wherein the primer pair consists of the oligonucleotide consisting essentially of SEQ ID NO:1 and the oligonucleotide consisting essentially of SEQ ID NO: 2.
15. The method of claim 10, wherein the amplification of the synthesized DNA is detected by Southern blot hybridization assay.
16. The method of claim 15, wherein the synthesized DNA is detected in the Southern blot hybridization assay with a probe consisting essentially of the oligonucleotide of SEQ ID NO:3.
17. A method for detecting the presence of a retrovirus in a biological sample comprising the steps of:
 - a) contacting the biological sample with a region of the encephalomyocarditis virus genome as an RNA template and a complementary DNA primer under conditions whereby the RNA template and the DNA primer will anneal and a DNA strand will be synthesized as an extension from the DNA primer if reverse transcriptase is present in the sample;

b) amplifying the synthesized DNA by the polymerase chain reaction method whereby the conditions for the amplification comprise 30-40 cycles of heating the synthesized DNA and a primer pair to 93 to 96°C for 30 seconds to one minute, 53 to 56°C for 30 seconds to one minute and 70 to 74°C for 30 seconds to five minutes; and

c) detecting the amplification of the synthesized DNA, the amplification of the synthesized DNA indicating the presence of reverse transcriptase in the biological sample, thus indicating the presence of a retrovirus in the biological sample.

18. The method of claim 17, wherein the complementary DNA primer is the oligonucleotide of SEQ ID NO:2.

19. The method of claim 17, wherein the amplification of the synthesized DNA is detected by Southern blot hybridization assay.

20. The method of claim 19, wherein the synthesized DNA is detected in the Southern blot hybridization assay with a probe consisting essentially of the oligonucleotide of SEQ ID NO:3.

21. The method of claim 17, wherein the RNA template is the ribonucleotide of SEQ ID NO:4.

22. The method of claim 21, wherein the conditions for the amplification of the DNA comprise about 35 cycles of heating the synthesized DNA and a primer pair to about 95°C for one minute, about 55°C for one minute and about 72°C for one minute.

23. The method of claim 22, wherein the primer pair consists of the oligonucleotide consisting essentially of SEQ ID NO:1 and the oligonucleotide consisting essentially of SEQ ID NO: 2.

24. A method of detecting the presence of a retrovirus in a biological sample comprising the steps of:

a) contacting the biological sample with a ribonucleotide of SEQ ID NO:4 and an oligonucleotide of SEQ ID NO:2 under conditions whereby the oligonucleotide and the ribonucleotide will anneal and a DNA strand will be synthesized as an extension from the oligonucleotide if reverse transcriptase is present in the sample;

b) amplifying the synthesized DNA by the polymerase chain reaction whereby the conditions of the amplification comprise about 35 cycles of heating the synthesized DNA and a primer pair consisting of the oligonucleotide consisting essentially of SEQ ID NO:1 and the oligonucleotide consisting essentially of SEQ ID NO:2 to about 95°C for one minute, about 55°C for one minute and about 72°C for one minute; and

c) detecting the amplification of the synthesized DNA, the amplification of the synthesized DNA indicating the presence of reverse transcriptase in the biological sample, thus indicating the presence of a retrovirus in the biological sample.

25. The method of claim 24, wherein the amplification of the synthesized DNA is detected by Southern blot hybridization assay.

26. The method of claim 25, wherein the synthesized DNA is detected in the Southern blot hybridization assay with a probe consisting essentially of the oligonucleotide of SEQ ID NO:3.

27. A kit for detecting the presence of a retrovirus in a biological sample, comprising a suitable region of the encephalomyocarditis virus genome as an RNA template and a complementary DNA primer for reverse transcriptase and a primer pair for polymerase chain reaction.

28. The kit of claim 27, wherein the complementary DNA primer is the oligonucleotide of SEQ ID NO:2.
29. The kit of claim 27, wherein the RNA template is the ribonucleotide of SEQ ID NO:4.
30. The kit of claim 27, wherein the primer pair consists of the oligonucleotide consisting essentially of SEQ ID NO:1 and the oligonucleotide consisting essentially of SEQ ID NO: 2.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 96/01257

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12Q 1/48, C12Q 1/70, C12Q 1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12Q, C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPOQUE, PAJ, MEDLINE, BIOSIS, DERWENT BIOTECH ABSTRACTS, CA, SCISEARCH, PATENT CITATION INDEX, WPI, EMBL/SWISSPROT/ GENBANK/ DDBJ (STRAND)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO, A1, 9323560 (SCHÜPBACH, JÖRG), 25 November 1993 (25.11.93), page 21, line 5 - line 28; page 102 - page 108, claim 1 --	1-30
D,X	Medical Sciences, Volume 91, February 1994, Pyra Halina et al, "Ultrasensitive retrovirus detection by a reverse transcriptase assay based on product enhancement", page 1544 - page 1548, see whole document --	1-30

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

- * Special categories of cited documents
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed
- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search

13 May 1996

Date of mailing of the international search report

26.06.96

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 96/01257

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>Dialog Information Service, file 154, Medline, Dialog accession no. 08010946, Medline accession no. 92148946, Duke GM et al: "Sequence and and structural elements that contribute to efficient encephalomyocarditis virus RNA translation", J Virol (UNITED STATES) Mar 1992, 66 (3) p1602-9 + Emurl: ID=EMCPOLYP AC=M81861</p> <p>-- -----</p>	<p>2,6,9,14,16, 18,20,23,24, 25,26,28,30</p>

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

01/04/96

PCT/US 96/01257

Patent document
cited in search reportPublication
datePatent family
member(s)Publication
date

WO-A1- 9323560

25/11/93

NONE